likely, nocturnally active D_8 mice with an evening high in audiogenic abnormality may be counterposed to certain types of "morning-fitters" among diurnally active human beings.

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3. This investigation was aided by the Elsa U. Pardee Foundation and by research grants from the U.S. Public Health Service, the Minnesota Department of Public Welfare, and the American Cancer Society.
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Agar Substrates for Study of Microepidemiology and Physiology in Cells in vitro

Despite the general usefulness of monolayer cell and tissue cultures on glass, there are experimental situations in which multilayered systems are desirable. Certain shortcomings in monolayer cellular systems for the study of infectious disease are apparent, particularly if a succession of cells must be parasitized to obtain full development of infection or to demonstrate the resistance of cells. The relatively great expanse of cells and fluid nutriments permits exposure of sensitive agents to inhibitory components in the liquid phase. There is loss of viral and bacterial agents and of infected cells during renewal of the supernates (*1*). In the case of microorganisms which can proliferate independently, streptomycin (*2*) or other extracellular inhibitors must be added to ensure an intracellular type of infection.

Combinations of infectious agents with cells or cell colonies on agar provide a contiguity of cells which is closer than that in animal tissues; extracellular inhibitors are of less concern; neither the agent nor the cells can escape the experimental arena. Many microepidemiological problems, therefore, may be studied more advantageously than in the whole animal or in monolayer cell cultures.

The human tubercle bacillus, strain H-37Rv, grows in cell colonies of Earle's L strain of mouse fibrocytes and Gey's strain HeLa. The failure of *Mycobacterium lepraemurium* to achieve more than the usual 2.5- to 3-fold multiplication (*3*) reveals that the problems of this organism are more complicated than the simple need for continuous existence within host cells.

The reservoirs of nutriment provided in the agar systems to be described have also permitted the maintenance of cell cultures for extended periods with minimal care and have proved useful in the study of organized tissue fragments (see also *4*).

Agar substrates were prepared by combining at 50°C double-strength nutriments with equal volumes of 2 to 4 percent purified agar (*5*) in BSS just prior to the preparation of plates, impregnated filter paper strips (*6*), or agar slants (*7*). Plates and impregnated papers were inoculated with cells or tissue fragments without prior drying, while agar slants to carry circumscribed cell colonies were dehydrated in inverted cotton-stoppered tubes overnight at 37°C. Cell inocula were standardized by transferring 2-mm loops (0.0025 ml) from dense, enumerated suspensions of strain L and strain HeLa. Several assemblies of agar systems which provide a reservoir of renewable, slowly available liquid nutriments are shown in Fig. 1.

The development of large bacterial colonies after 24 to 72 hours on agar indicates that diffusion rates are more than adequate for tissue cells. On slants containing 3 ml of serum, 2 percent, Eagle's supplement, and 0.5 percent Bacto-peptone (*3*) without liquid phase, strain L produced relatively thick colonies within 2 weeks at 34°C. Cells at the margins of such colonies were round and clear. If 0.5 ml of liquid phase (1/7 of the system) was replaced each 2 weeks, very thick mucoid colonies developed within 2 months. In such media supplemented with pyruvate (50 mg/100 ml) and citrate (0.03 percent) the maintenance of uniformly high viability after 3 months was shown by exclusion of eosin (*8*) and by vigorous growths following transfers to new media.

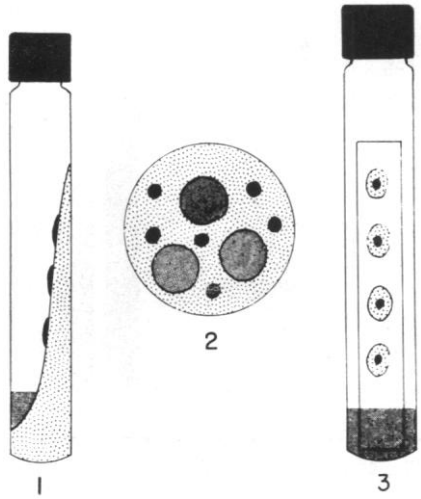

Fig. 1. Agar systems with diffusible nutriments: 1, agar slant; 2, agar plate with three "wells"; 3, agar-impregnated filter paper carrying four cell colonies.

Approximately 5000 L cells were required per site for reliable inoculation of the medium mentioned above. Although incubation in oxygen tensions of 1 to 4 percent, or in the presence of 20 percent serum, decreased the required inoculum, uniform growth was not obtained from less than 2000 cells. Growth of strain HeLa has been initiated with as few as two cells.

Quantitative samples are recoverable from plates or slants by the application of 0.25 percent pancreatin to each cell colony, and by several rinses with known volumes of diluent. Cells are recovered quantitatively from single colonies on plates, slants, or impregnated papers by first removing a disc of agar which includes the colony, and then by use of the foregoing procedure. A bacteriological wire transfers sufficient cells for quantitative determinations of cell types and ratios, the proportion of cells infected, the percentage of cells capable of excluding eosin, and for transfers of viable inoculum.

Fragments of guinea pig bone marrow were explanted on agar slants, plates, and impregnated papers containing 10 percent guinea pig serum and the aforementioned supplements. After 24 hours the migrating cells produced 5- to 10-mm halos in which neutrophils preponderated. The cells within the explants retained typical morphology and staining characteristics for 2 to 3 days. Following the death or modification of various cell types, or both, and after 4 weeks, some 20 to 30 percent of the cells in explants plus deteriorating halos were viable (*9*).

Continuous ciliary beating was observed on the surface of fragments of embryonic chick and embryonic and adult human bronchial tissues during at least two weeks on agar substrates containing 25 percent chicken or human serum and the supplements mentioned (*9*).

It seems, therefore, that cell colonies and tissue fragments on agar substrates are useful for the study of many problems in infectious disease and cellular physiology or differentiation, and also for the maintenance of cell lines with minimal effort (*10*).

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10. The assistance of Miss Jean M. McCarthy is gratefully acknowledged.

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Loss of Precipitating Activity of Antibody without Destruction of Binding Sites

In investigating the composition of the specific combining region of antibody, we have studied the reaction of acetic anhydride with rabbit antibody homologous to the *p*-azobenzoate group. Others have found that acetylation of various antibodies results in loss of ability to precipitate with antigen (*1*). Marrack and Orlans (*2*) reported that when rabbit antibodies against several different antigens were acetylated, they would no longer precipitate, but that a large fraction of each acetylated antibody coprecipitated when added to a mixture of antigen and untreated antibody. They concluded that the failure to precipitate was due either to electrostatic repulsion among protein molecules or to deformation of the antibody molecule resulting from the increased negative charge which accompanies acetylation of amino groups.

In the present work, acetylated preparations of rabbit antibody homologous to the *p*-azobenzoate group were tested for ability to precipitate an ovalbumin-*p*-azobenzoate test antigen, and also to bind homologous hapten [by equilibrium dialysis (*3, 4*)]. Binding experiments provide direct information bearing on the number of combining sites present. It was found that mild acetylation results in complete loss of precipitating activity but that the ability to bind homologous hapten was affected only slightly. The results confirm the hypothesis of Marrack and Orlans and show that an antibody with both combining sites intact may fail to precipitate. Another conclusion is that the nonspecific electrostatic effect in the interaction of this antibody with the negatively charged homologous hapten is small.

Experiments were carried out with antisera obtained from ten rabbits repeatedly injected with a bovine γ-globulin-*p*-azobenzoate antigen, prepared by coupling 30 mg of diazotized *p*-aminobenzoic acid to 1 g of bovine γ-globulin (*4, 5*). The γ-globulin fraction of the pooled antisera was separated by precipitation with sodium sulfate (*6*). Free electrophoresis showed the presence of only one peak, corresponding to γ-globulin. Two 5 ml portions of the globulin (32.4 mg/ml) were acetylated at 0°C by addition, with a microburette, of several portions of acetic anhydride over a period of 15 minutes. The amounts of the reagent used in each experiment are given in Table 1. The mixture was stirred continuously and allowed to react for 1 hour; *p*H was maintained at 8.0 to 8.5 with 0.1*N* NaOH. An untreated control and the acetylated samples were dialyzed overnight against 10 liters of saline-borate buffer at 3° to 5°C and adjusted to the same final protein concentration.

The results are shown in Table 1. The unacetylated globulin and sample A (about 20 percent of the amino groups acetylated) exhibited typical precipitin curves. However, the entire curve for A was displaced downward and, as is indicated in the table, only 41 percent as much precipitate formed at the optimum. No precipitation or turbidity was observed in mixtures of antigen and sample B, which was more extensively acetylated (85 percent of amino groups reacted). In contrast to these results, it is evident (Table 1) that the ability to bind the homologous hapten, *p*-iodobenzoate-I^{131} (*7*) was not greatly affected by acetylation. There was no appreciable difference in the concentration of hapten bound by the control or sample A. In the case of sample B, which formed no precipitate, the concentration of hapten bound, corrected to the same free hapten concentration (*8*), was 79 percent of that bound by the unacetylated sample. This small adverse effect of acetylation on binding may be attributed either to a decrease in the average combining constant (K), the loss of effective combining sites, or both. Some decrease in K appears likely since the hapten is negatively charged and the net negative charge per molecule in preparation B was increased by about 65 units on acetylation.

We may obtain an estimate of the maximum number of combining sites affected by assuming that K was unchanged and that the observed effects on binding were due entirely to the attack of acetic anhydride on groups in the specific combining region of the antibody. It would appear then that a maximum of about one-fifth of the sites or somewhat less than two-fifths of the molecules were affected [assuming random acetylation and the presence of two combining sites per molecule (*9*)]. It is also possible that a smaller number of sites were affected and that a decrease in K was partly or entirely responsible for the reduced binding. In any event, the data show that complete loss of precipitability may occur without a corresponding decrease in the number of sites capable of combining with hapten.

The results suggest that the failure of the acetylated antibody to precipitate is attributable to a factor other than attack of the reagent on the specific combining region. It is possible that the acetic anhydride reacts with a group in a part of the combining site which interacts with antigen but lies beyond the region making contact with *p*-iodobenzoate (*10*). However, this alternative cannot apply to the data of Marrack and Orlans (*2*), who used only protein antigens. Their explanation, that acetylated antibody does not precipitate because of electrostatic repulsion resulting from its increased negative charge, can also account for the lack of precipitation in the antiazobenzoate system (*11*). The interaction of

Table 1. Effects of acetylation on the γ-globulin fraction of rabbit anti-*p*-azobenzoate antiserum. Mean deviations given in table are based on duplicate or triplicate analyses. The procedure for acetylation is described in the text.

Sample	Ac_2O used (mg)	No. of amino groups per molecule remaining*	Antibody precipitable by antigen† (μg)	Labeled *p*-iodobenzoate bound‡ ($\times 10^6$M)
Normal globulin	0	–	–	0.21 ± 0.02
Antibody globulin (control)	0	75 ± 8§	228 ± 2	7.5 ± 0.2
Antibody globulin (A)	2.8	58 ± 1.5	93 ± 3	7.2 ± 0.8
Antibody globulin (B)	14	11 ± 1.5	0‖	6.6 ± 0.1

* From van Slyke amino nitrogen analyses. Calculation based on molecular weight 160,000.
† Precipitin reaction with 0.20 ml each of antibody and antigen. The latter was prepared by coupling 60 mg of diazotized *p*-aminobenzoic acid to 1 g of ovalbumin. Values given are for optimum antigen concentration.
‡ Protein concentration, 16.2 mg/ml. Free hapten concentration 1.71 ± 0.06, 1.75 ± 0.07 and 1.90 ± 0.17 × $10^{-6}M$, corresponding to control and samples A and B; and 2.95 ± 0.07 × $10^{-6}M$ for the normal globulin. Values for the antibody preparations are corrected for the small amount of binding by normal globulin.
§ Amino acid analysis (*12*) indicates the presence of 72 free amino groups per molecule.
‖ No turbidity or precipitation observed.